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(71) Applicant: BRIGHAM AND WOMEN'S HOSPITAL, INC. [US/US]; 75 Francis Street, Boston, MA 02115 (US).		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: TZIANABOS, Arthur, O.; 37 Hampshire Road, Reading, MA 01867 (US). ONDERDONK, Andrew, B.; 28 Lynn Terrace, Westwood, MA 02090 (US). KASPER, Dennis, L.; 544 Ward Street, Newton Center, MA 02159 (US).			
(74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).			
(54) Title: CAPSULAR POLYSACCHARIDE IMMUNOMODULATOR			
(57) Abstract  Methods and products for protecting against abscess formation associated with surgery, trauma or diseases that predispose the host to abscess formation are provided. Methods for forming immunomodulators and pharmaceutical compositions relating thereto are also provided. The products useful in the invention are polysaccharides including a repeat unit having a positively charged free amino group and a negatively charged group. The preferred polysaccharide is <i>B. fragilis</i> capsular polysaccharide A.			

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## CAPSULAR POLYSACCHARIDE IMMUNOMODULATOR

This invention relates to immunomodulators and methods for protecting a subject against abscess formation associated with bacterial infection or contamination.

A commonly occurring complication associated with leakage of colonic bacteria into the peritoneum is intra-abdominal sepsis and abscess formation. An abscess is an encapsulated collection of bacteria, lymphocytes, macrophages, polymorphonuclear leukocytes and fibrin that forms in response to bacterial insult or contamination within the peritoneal cavity, such as occurs during a surgical procedure, trauma or diseases such as appendicitis or cancer. Invasion of the exposed body area by the bacteria may occur in a localized area within the peritoneal cavity, retroperitoneal space, pelvis or other spaces or organs in the body. The infected tissue area remains relatively immune to antibiotics which are unable to penetrate the tissue structures and effectively clear walled-off bacteria. If the abscess is left untreated, it may cause fever, prolonged hospitalization, and in some cases mortality. If the abscess ruptures, it will release its bacterial contents into the peritoneal cavity, which can in turn lead to recurring sepsis in these patients. Currently when abdominal surgeries are performed, antibiotics are administered prophylactically as well as post-operatively. However, once an abscess has formed, the major course of action is further surgical intervention to drain the offending abscess, a time-consuming and costly procedure.

It has been impractical to immunize patients against abscess formation such as for intra-abdominal surgery because there simply are too many strains of bacteria capable of causing abscess formation, and protection against one does not confer protection against another. It also is unsettled whether vaccination and consequent induction of an immune response would confer adequate protection against abscess formation by any particular bacterium. There also exist problems and dangers associated with administering live or attenuated strains of bacteria to humans, further discouraging efforts to produce vaccines containing a large number of different bacteria.

Capsular polysaccharides of bacteria can be found covering the surface of some bacteria pathogenic to humans. Polysaccharides have been characterized as T cell-independent antigens that elicit only humoral antibody responses. Although many polysaccharides have been shown to be immunogenic, some are only weakly immunogenic at best.

Bacteroides fragilis is a predominant obligate anaerobe isolated from intra-abdominal abscesses. The capsular polysaccharide complex (CPC) has been identified as the region of B. fragilis which causes abscess formation. This carbohydrate complex covers the surface of

B. fragilis. The isolated complex alone can interact with the host immune system, in the presence of adjuvant (sterile cecal contents and barium sulphate), to elicit a patho-biologic response that results in fully formed intraperitoneal abscesses in individuals injected intraperitoneally with the complex. Studies were performed in rodent models in which B. fragilis or its CPC were injected intra-peritoneally. Both intact B. fragilis and CPC alone provoked abscess formation associated with intra-abdominal sepsis.

It was investigated whether the CPC of B. fragilis, in the presence of Freund's incomplete adjuvant, could be used to immunize subjects against subsequent infection and abscess formation by B. fragilis. It was by no means predictable that this would be possible based upon the property of CPC alone to provoke abscess formation since "immunity" and abscess formation are not known to result from remotely related immunological responses. When CPC was administered subcutaneously it was found to confer immunological protection against intraperitoneal CPC-mediated abscess induction in a rat model. Protection against abscess formation by this polysaccharide complex was determined to be mediated by a T cell-dependent host response.

Although subcutaneous administration of either B. fragilis or CPC is sufficient to protect animals against abscess formation subsequent to challenge with B. fragilis or CPC, neither conferred immunity against other bacterial strains, as was expected. They therefore have no use as a "vaccine" for abscess formation caused by the multitude of organisms normally found in the colon.

The CPC consists of two distinct high molecular weight polysaccharides, termed A and B. Each polysaccharide is composed of distinct oligosaccharide repeating units possessing uncommon constituent sugars with free amino, carboxyl and phosphonate groups. Polysaccharide A (PSA) has a tetrasaccharide repeating unit with a balanced positively charged amino group and negatively charged carboxyl group. Polysaccharide B (PSB) has a hexasaccharide repeating unit, including an unusual 2-amino ethylphosphonate substituent containing a free amino group and negatively charged phosphate group. The galacturonic acid residue contains an additional negatively charged carboxyl group. Ionic interaction between the two saccharide chains tightly links PSA and PSB into the high molecular weight CPC complex. The complex capsular motif is a conserved trait for all strains of B. fragilis that have thus far been examined.

It would be extremely desirable to have a pharmaceutical preparation that could protect a host organism against abscess formation associated with infection by multiple bacterial strains.

#### Summary of the Invention

Methods and products for protecting against abscess formation associated with surgery,

trauma or diseases that predispose the host to abscess formation are provided. Methods for forming immunomodulators and pharmaceutical compositions relating thereto also are provided.

It has been discovered that polymers having a particular structural motif can protect animals against challenge with abscess-inducing bacteria. This motif includes a positively charged free amino group and a negatively charged group on a repeating unit. Such polymers are capable of inducing "cross-protection". That is, a single polymer can produce protection against abscess formation by a variety of bacteria.

A method for inducing protection against abscess formation associated with surgery, trauma or diseases that predispose the host to abscess formation is provided. A pharmaceutical preparation is administered to a subject in conjunction with intra-abdominal surgery or upon presentation of a predisposing condition. The preparation includes an effective amount for inducing protection against abscess formation of a polymer of repeating units of a charge motif characteristic of PSA of B. fragilis, the motif being a positively charged free amino moiety and a negatively charged moiety selected from the group consisting of carboxyl, phosphate, phosphonate, sulfate and sulfonate. The polymer can be a polysaccharide formed of repeating units of a maximum of ten saccharides. Preferably the negatively charged moiety is carboxyl, phosphate or phosphonate. The polymer is free from complexation as part of a B. fragilis CPC.

Preferably the polysaccharide is formed of repeating units of a maximum of five monosaccharides. Such polysaccharides occur in nature and may be isolated. One such polysaccharide, the most preferred, is capsular PSA of the B. fragilis CPC. In nature PSA occurs only in complexed form, tightly bound to the B. fragilis capsular PSB. The A:B CPC is not capable of inducing cross-protection to infection with other bacteria. Thus, the invention contemplates administration of isolated capsular PSA, free from complexation as part of a B. fragilis CPC.

The polysaccharides useful according to the invention also may be synthesized from naturally occurring polysaccharides that do not possess the requisite motif. For example, certain naturally occurring polysaccharides have a negatively charged group and at least one N-acetyl moiety on each repeating unit. Such polysaccharides may be de-N-acetylated to convert the N-acetyl moiety to a free amino moiety, thereby creating the necessary structural motif. Other naturally occurring polysaccharides include imine groups which can be reduced to form a free amino moiety, thereby creating together with a negatively charged group the necessary structural motif.

Thus, the invention contemplates methods for preparing pharmaceuticals by selecting polymers such as polysaccharides having repeating units of a maximum of ten saccharides, each unit having at least one negatively charged moiety, preferably being carboxyl, phosphate, phosphonate, sulfonate or sulfate. Each repeating unit also includes a moiety that may be modified to form a free amino moiety. Such modified polymers then are mixed with pharmaceutically acceptable carriers, preferably in amounts to form effective doses for protecting a subject against abscess formation associated with surgery, trauma or diseases that predispose the host to abscess formation.

Pharmaceutical preparations also are provided. The pharmaceutical preparations include a polymer such as a modified polysaccharide formed of repeating units of a maximum of ten units, each repeating unit including a free amino moiety and a negatively charged moiety, preferably being carboxyl, phosphate, phosphonate, sulfonate or sulfate, together with a pharmaceutically acceptable carrier. The polymer is free from complexation as part of a B. fragilis CPC. Preferably the polymer is a bacterial polysaccharide and most preferably the polysaccharide is a component of the B. fragilis CPC. PSA may also be modified, for example, to contain a hydroxymethyl group.

Exemplary pharmaceutical preparations include monomer repeat units that carry both the free amino group and the negatively charged group, dimer repeat units in a 1-4 linkage wherein the negatively charged group is on the first saccharide, trimer repeat units wherein the negatively charged group is on the first saccharide, wherein the free amino moiety is also on the first saccharide and/or wherein the third saccharide is free of any amino or negatively charged moiety, and tetramer repeat units with a trimeric backbone, preferably characteristic of B. fragilis PSA.

The products and methods of the invention may be administered with immunomodulators and/or antimicrobials, separately or as a part of the pharmaceutical preparations of the invention. Preferred immunomodulators are those that enhance protection against abscess formation. Useful immunomodulators include adjuvants; certain cytokine blockers; and certain cytokines. Thus, the invention involves pharmaceutical preparations containing such immunomodulators and/or antimicrobials together with the polymers useful according to the invention. This includes naturally occurring bacterial polysaccharides that previously may have been used as immunogens to stimulate a humoral B cell response, but have not before been used to protect against abscess formation and have not been used together with immunomodulators and/or antimicrobials (e.g. Streptococcus pneumoniae polysaccharide, Trypanosoma cruzi lipopeptidophosphoglycan and Pseudomonas aeruginosa Fisher immunotype 1 O-antigen).

The methods and products of the invention are useful in protecting against abscess formation associated with infection by many different microorganisms, including microorganisms selected from the group consisting of Bacteroides, Clostridia, Fusobacteria, Enterococcus, Prevotella, Porphyromonas, Staphylococcus, Propionobacteria, Peptostreptococcus, Streptococcus, Veillonella, Actinomycetes, and Escherichia coli. The pharmaceutical preparations may be administered before and/or after exposure to the abscess-forming conditions. Parenteral administration is preferred.

It is believed that bacterial polysaccharides of the invention most likely act to stimulate T cell proliferation, which has not before been described in connection with bacterial polysaccharides. The invention avoids the dangers associated with using live bacteria to stimulate an immune response and further provides cross-protection against a variety of strains of bacteria. The invention further can be used in connection with both planned and emergency surgeries, trauma or diseases that predispose the host to abscess formation. The invention is described in greater detail below.

#### Detailed Description of the Invention

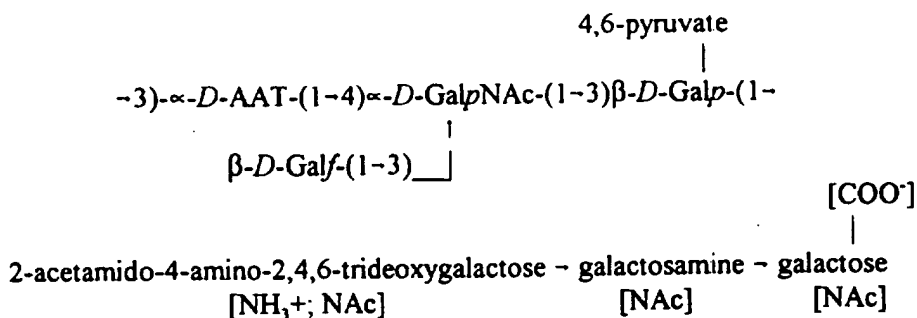
The invention is useful whenever it is desirable to prevent bacterial abscess formation in a subject. This includes prophylactic treatment to prevent such conditions in planned surgical procedures as well as emergency situations. Elective surgeries include the following intra-abdominal surgeries: right hemicolectomy; left hemicolectomy; sigmoid colectomy; subtotal colectomy; total colectomy; laparoscopic or open cholecystectomy (gall bladder); gastrectomy; etc. Emergency surgeries include those to correct the following conditions: perforated ulcer (duodenal or gastric); perforated diverticulitis; obstructive diverticulitis; perforated appendicitis; blunt abdominal trauma; penetrating abdominal trauma; second operation to drain abscess; etc. The invention also is useful with nonintra-abdominal surgeries such as cardiac surgeries and surgeries to correct wound infections. The invention also is useful in connection with diseases that predispose a subject to abscess formation such as pelvic inflammatory disease, inflammatory bowel disease, urinary tract infections and colon cancer. The invention thus is useful with abscesses of virtually any tissue or organ, including specifically but not limited to dermal abscesses such as acne. Those of ordinary skill in the art to which this invention pertains will recognize the range of conditions and procedures with which the invention is useful. A subject as used herein means: humans, primates, horses, cows, sheep, pigs, goats, dogs, cats, and rodents.

It has been discovered that certain polymers can be used to stimulate host T cells and induce protection against numerous bacteria. This protective effect is T cell-dependent and not

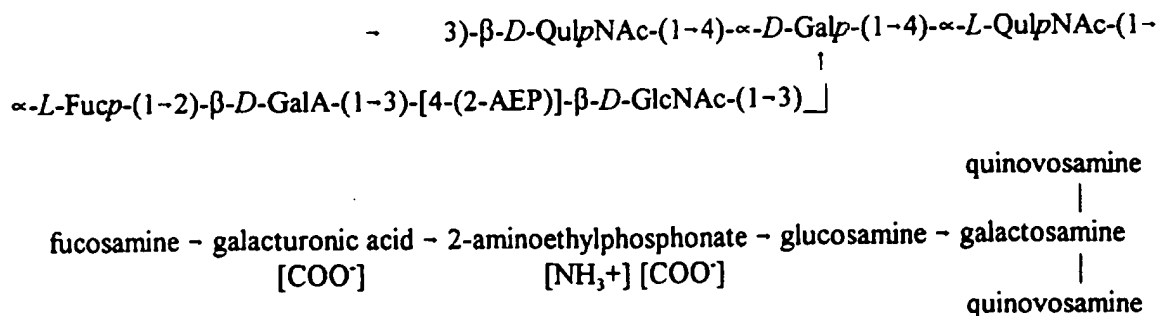
mediated by a humoral antibody response. As such, administration of the preparations of the invention is not "vaccination" and the preparations are not "vaccines" which mediate protection that is specific to bacteria expressing the immunizing antigen.

It was discovered that the B. fragilis capsular PSA, when separated from the polysaccharide A:B complex, was capable of conferring broad protection against abscess formation resulting from challenge with B. fragilis and other bacterial species. This was unexpected because PSA does not exist except as a complex with PSB in nature, and it therefore was not predictable that PSA would have any activity when separated from PSB. What is more surprising is that this protection extends to abscess formation resulting from infection by organisms other than B. fragilis because PSA does not exist on bacteria other than B. fragilis. Thus, the preparations of the invention represent the first "universal" immunomodulators capable of protecting against abscess formation that might result from infection/contamination by any number of bacteria. The invention thereby opens the door to pretreating abdominal surgical patients, trauma patients or patients with diseases that predispose the host to abscess formation with a safe immunomodulator to provoke a generalized immune response to protect against abscess formation.

The protective effect described above was seen also using purified B. fragilis PSB, isolated and separated from PSA. PSA has the following structure:



PSB has the following structure:





It was determined that there are particular structural features on PSA and PSB which mediate their ability to induce intra-abdominal abscesses. Chemical neutralization or removal of the charged amino or carboxyl group abrogated abscess induction by these polysaccharides. Polysaccharides from other organisms, such as the group antigen or capsular polysaccharide from S. pneumoniae type 1 strains, that had different repeating unit structures but the same charged structural groups (i.e., at least one free amino and one negatively charged group) also promoted abscess formation. Both the positively and negatively charged groups on these polysaccharides also modulate their ability to induce abscess formation and to protect animals against abscess formation. Treatment with either PSA or PSB protected animals against abscess formation subsequent to challenge with PSA, PSB or S. pneumoniae type 1 capsular polysaccharide. Both the positive and negative charges on PSA are essential to the ability of this polymer to confer protection against abscess formation, as neutralization of either charge abrogated the protection. The ability of PSA and PSB to confer protection against abscess formation is mediated by T cells.

Polysaccharides useful according to the present invention include those naturally occurring ones that include the requisite charged groups. These may be derived from bacterial sources. Bacteria used as starting materials to obtain capsular polysaccharides can be obtained commercially from a number of sources. For example, the B. fragilis NCTC 9343 and ATCC 23745 may be obtained from the National Collection of Type Cultures (London, England) and the American Type Culture Collection (Bethesda, MD). PSA and PSB can be purified from the above bacteria following the protocol of Pantosti et al., Infection and Immunity 59:2075-2082 (1991).

In addition to the naturally occurring polysaccharides, those that consist of a repeating unit containing at least one N-acetyl sugar and at least one uronic acid (sugar with a negatively charged carboxyl group) can be modified to produce the immune response of the invention. A poly-saccharide repeating unit containing at least one N-acetyl sugar and at least one uronic acid can be de-N-acetylated to create a free amino group yielding a polysmer with the correct charge motif. Molecules which may be de-N-acetylated include S. typhi capsular polysaccharide (Vi antigen), E. coli K5 capsular polysaccharide, Staphylococcus aureus type 5 capsular polysaccharide, Group B Streptococcus type 3 capsular polysaccharide, and Rhizobium meliloti exopolysaccharide II.

Bacterial polysaccharides which possess imine groups (C=NH) in addition to free carboxyl groups may be modified and used according to the invention. Many of the P. aeruginosa O-specific side chains possess imine groups. Imine groups can be reduced with sodium

borohydride ( $\text{NaBH}_4$ ) to create free amino groups ( $\text{NH}_3^+$ ). An example of a compound which may be reduced with sodium borohydride to create free amino groups is

P. aeruginosa Fisher 7.

One important polysaccharide antigen is the capsular PSA from B. fragilis, modified slightly, first by oxidation with 0.01 M Sodium metaperiodate ( $\text{NaIO}_4$ ) to selectively create a carbonyl group ( $\text{C}=\text{O}$ ) on the galactofuranose side chain of each repeating unit (Paoletti et al., Journal of Clinical Investigation 89:203-209 (1992)), and then by reaction with a reducing agent such as sodium borohydride to convert the carbonyl group to a hydroxymethyl group ( $\text{CH}_2\text{OH}$ ).

The size of the polymer useful according to the invention varies greatly. Polysaccharides between 500 and 20,000,000 daltons will be typical. The polysaccharides useful in the invention also may be delivered in mixtures of different polysaccharides.

Naturally occurring polysaccharides can be modified to yield immunomodulators useful in the invention. S. typhi has a capsular polysaccharide (Vi antigen) that is formed entirely of repeating monomers of galactosaminuronic acid. This acid includes a carboxylic moiety and an N-acetyl moiety. The N-acetyl moiety can be modified to yield a free amino group such that each monomeric repeating unit then has both a positively and negatively charged group.

Polysaccharides that are complexes exist and can be modified to yield immunomodulators useful in the invention. E. coli K5 capsular polysaccharide is formed of repeat units of a complex of glucuronic acid and glucosamine linked together in 1-4 linkages. The glucuronic acid carries a carboxylic acid moiety and the glucosamine carries an N-acetyl group, which can be modified to form a free amino group. When so modified a complex repeat unit having both a negatively charged moiety (on the first sugar) and a free amino group (on the second sugar) is formed.

Polysaccharides that are composed of trimeric repeating units exist and can be modified to yield immunomodulators useful in the invention. S. aureus type 5 capsular polysaccharide is formed of repeat units of a trimer of mannosaminuronic acid - fucosamine - fucosamine. The mannosaminuronic acid carries a carboxylic acid moiety and the fucosamines carry N-acetyl moieties which can be modified to form free amino moieties. When so modified, a trimeric repeat unit having a negatively charged moiety (on the first sugar) and at least one positively charged moiety (on the second and third sugars) is formed. In a similar manner, P. aeruginosa O-antigens can be modified to yield immunomodulators useful in the invention. Examples include trimers that carry carboxylic acid moieties and imine moieties which can be modified to yield free amino groups. Fisher immunotype 7, Lanyi-Bergan O2a, O2b and Lanyi-Bergan O2d, and 2f have polysaccharides formed of trimeric repeat units with carboxylic acid moieties on the first and

second sugars and an imine moiety on the first sugar. (The third sugar is free of a charged moiety; all sugars also carry an N-acetyl moiety). For example, the first sugar can be modified so as to carry both a free amino moiety and the carboxylic acid moiety. Likewise the N-acetyl groups could be modified to yield a different arrangement useful according to the invention.

Polysaccharides that have longer repeat units such as tetramers and pentamers also can be modified as described above. It is believed that repeat units up to decimers are useful according to the invention. In addition, repeat units including side chain sugars also are useful, including those wherein one or both of the free amino and negatively charged moieties are located on such side chains. Furthermore, such side chains carrying the charged moieties need not be sugars, although more typically the backbone of the repeat unit is made up of only sugars.

It is preferred that the repeat unit have no more than three free amino groups, and preferably no more than two such groups. It also is preferred that there be at least one negatively charged group for each free amino group.

The starting materials further need not be derived from bacterial origin. Any polysaccharides, natural or synthetic, carrying carboxylic acid moieties and N-acetyl or imine groups may be modified as described above.

Specific examples together with chemical names and structural formulas are as follows. The invention, however, is by no means limited to the following examples. In the chemical formulas, use is made of the following abbreviations: AAT = 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; A = uronic acid; NAc = N-acetyl group; p = pyranose; AEP = 2-aminoethylphosphonate; COO<sup>-</sup> = carboxylate; and OAc = O-acetyl group. Polysaccharides having N-acetyl moieties:

*Salmonella typhi* capsular polysaccharide (Vi antigen)

$\alpha$ -(1-4)-D-GalANAc

galactosaminuronic acid

[COO<sup>-</sup>; NAc]

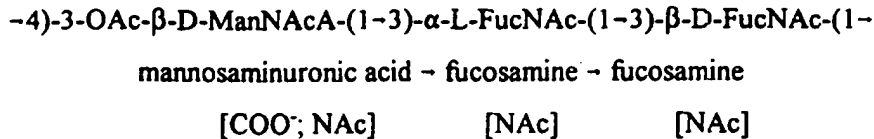
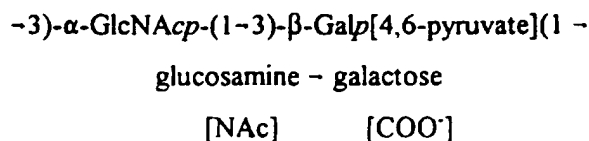
*Escherichia coli* K5 capsular polysaccharide

-4)- $\beta$ -D-GlcA-(1-4)- $\alpha$ -D-GlcNAc-(1-

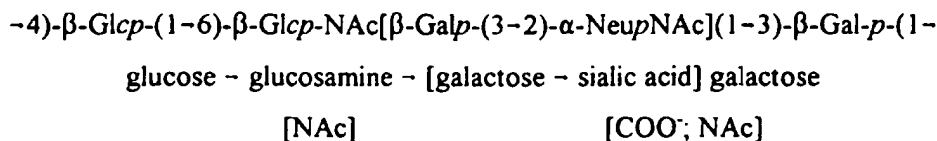
glucuronic ~ glucosamine ~ 1

[COO<sup>-</sup>] [NAc]

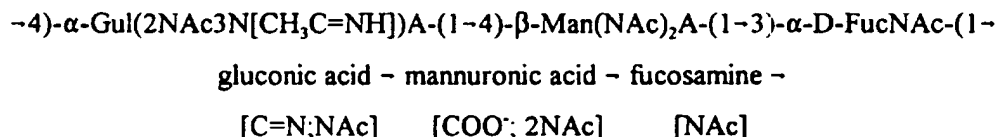
-10-

*Staphylococcus aureus* type 5 capsular polysaccharide*Rhizobium meliloti* exopolysaccharide II

## group B streptococcus type III capsular polysaccharide



Polysaccharide having an imine moiety:

*Pseudomonas aeruginosa* Fisher 7

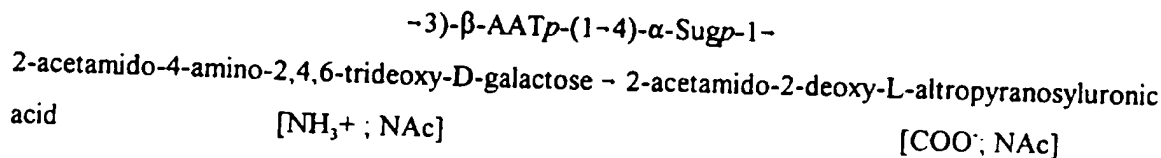
De-N-acetylation can be accomplished by conventional chemistry techniques well known to those of ordinary skill in the art. One suitable method involves the use of alkali with or without sodium borohydride. Twenty mg of polysaccharide is dissolved in 2M NaOH (3ml) and sodium borohydride is added (50 mg). The solution is heated to 100°C for 5 h. Following neutralization with acid, the solution is dialyzed against distilled water in the cold and freeze-dried. DiFabio, J. L., Michon, F., Brisson, J.R., Jennings, H.J., Wessels, M.R. Benedi, V.J., Kasper, D.L. Structure of the capsular polysaccharide antigen of type IV group B Streptococcus. 1989. Canadian Journal of Chemistry, 67:877-882.

For those polysaccharides that contain imine moieties (C-NH), free amino groups can be formed by conventional chemistry techniques known to those of ordinary skill in the art. One suitable method involves the use of sodium borohydride. The imine group can be reduced with sodium borohydride to create a free amino group. This is done by adding in excess of 5 mg of

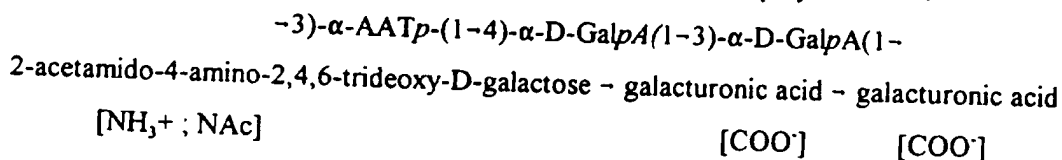
borohydride to polysaccharide dissolved in distilled water while stirring at room temperature for 2 hours. The mixture is then dialyzed against water and freeze dried. The reference from the reduction procedure above applies here as well.

Naturally occurring polysaccharides also may be used without modification in the methods of the invention and in forming the pharmaceutical preparations of the invention. Non-limiting examples are as follows:

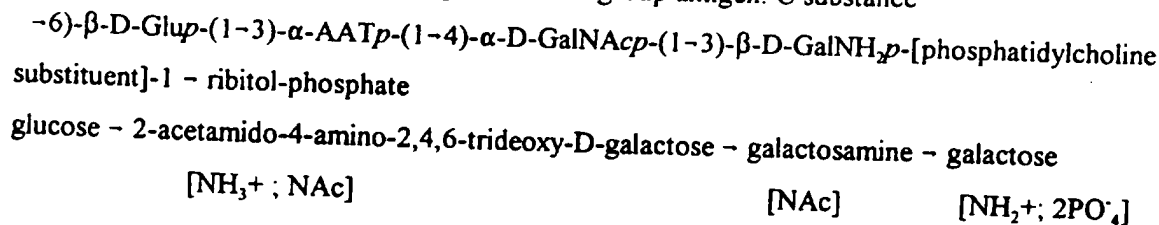
*Shigella sonnei* Phase I lipopolysaccharide O-antigen



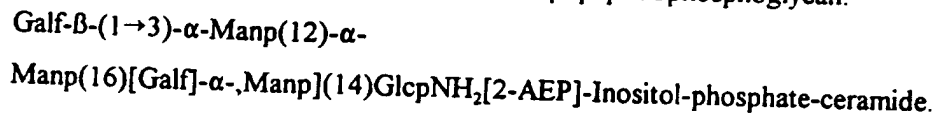
*Streptococcus pneumoniae* type 1 capsular polysaccharide



*Streptococcus pneumoniae* group antigen: C substance



A polysaccharide that does not have solely a sugar backbone but still is believed to be useful according to the invention is *T. cruzi* lipopeptidophosphoglycan:



The naturally occurring polysaccharides that may be used without modification also may be modified to selectively add, subtract or modify various moieties, including free amino moieties, negatively charged moieties or other moieties. Examples include adding free amino moieties by modifying existing N-acetyl groups or imine groups or forming hydroxymethyl groups from alcohol groups.

Polysaccharides useful according to the invention may be obtained from commercial sources or may be isolated and derived from natural sources such as bacteria, fungi, seaweed

and the like. The following is a list of bacterial polysaccharides and references which detail the isolation and preparation of such polysaccharides.

S. typhi capsule (Vi antigen), Szu, S.C., X. Li, A.L. Stone and J.B. Robbins, Relation between structure and immunologic properties of the Vi capsular polysaccharide, Infection and Immunity. 59:4555-4561 (1991).

E. coli K5 capsule, Vann, W., M.A. Schmidt, B. Jann and K. Jann, The structure of the capsular polysaccharide (K5 antigen) of urinary tract infective E. coli, 010:K5:H4. A polymer similar to desulfo-heparin, Eur. J. of Biochem. 116: 359-364, (1981).

S. aureus type 5 capsule, Fournier, J.-M., K. Hannon, M. Moreau, W.W. Karakawa and W.F. Vann, Isolation of type 5 capsular polysaccharide from S. aureus, Ann. Inst. Pasteur/Microbiol. (Paris). 138: 561-567, (1987).

R. meliloti expolysaccharide II, Glazebrook, J. and G.C. Walker, a novel expolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by R. meliloti, Cell. 65:661-672 (1989).

Group B streptococcus type III, Wessels, M.R., V. Pozsgay, D.L. Kasper and H. J. Jennings, Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B Streptococcus, J. of Biol. Chem. 262:8262-8267 (1987).

P. aeruginosa Fisher 7 O-specific side-chain, Knirel, Y.A., N.A. Paramonov, E.V. Vinogradov, A.S. Shashkov, B.A. N.K. Kochetkov, E.S. Stanislavsky and E.V. Kholodkova, Somatic antigens of P. aeruginosa The structure of O-specific polysaccharide chains of lipopolysaccharides of P. aeruginosa O3(Lanyi), 025 (Wokatsch) and Fisher immunotypes 3 and 7, Eur. J. of Biochem. 167:549, (1987).

S. sonnei O-specific side chain, Kenne, L., B. Lindberg and K. Petersson, Structural studies of the O-specific side-chains of the S. sonnei phase I lipopolysaccharide, Carbohydrate Research. 78:119-126, (1980).

S. pneumoniae type I capsule, Lindberg, B., Lindqvist, B., Lonngren, J., Powell, D.A., Structural studies of the capsular polysaccharide from S. pneumoniae type 1, Carbohydrate Research. 78:111-117 (1980).

S. pneumoniae group antigen, Jennings, H.J., C. Lugowski and N. M. Young, Structure of the complex polysaccharide C-substance from S. pneumoniae type 1, Biochemistry. 19:4712-4719 (1980).

The formulations of the invention are administered in pharmaceutically acceptable

solutions, which may contain pharmaceutically acceptable concentrations of salt, buffers, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

The capsular polysaccharide antigen may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonc, tartaric, citric, methane sulphonc, formic, malonic, succinic, naphthalene-2-sulphonc, and benzene sulphonc. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% W/V); citric acid and a salt (1-3% W/V); boric acid and a salt (0.5-2.5% W/V); and phosphoric acid and a salt (0.8-2% W/V). Suitable preservatives include benzalkonium chloride (0.003-0.03% W/V); chloro-butanol (0.3-0.9% W/V); parabens (0.01-0.25% W/V) and thimerosal (0.004-0.02% W/V).

The pharmaceutical compositions of the invention contain an effective amount of a polymer optionally included in a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid fillers, dilutants or encapsulating substances which are suitable for administration to a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the polymers of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Compositions suitable for parenteral administration conveniently comprise sterile aqueous preparations, which can be isotonic with the blood of the recipient. Among the acceptable vehicles and solvents are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for subcutaneous, intramuscular, intraperitoneal, intravenous, etc. administrations may be found in Remington's

Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

The term "adjuvant" includes any substance which is incorporated into or administered simultaneously with the polymer and which potentiates the immune response in the subject. Adjuvants include aluminum compounds, e.g., gels, aluminum hydroxide and aluminum phosphate, and Freund's complete or incomplete adjuvant (in which the polymer is incorporated in the aqueous phase of a stabilized water in paraffin oil emulsion). The paraffin oil may be replaced with different types of oils, e.g., squalene or peanut oil. Other adjuvants include BCG (attenuated Mycobacterium tuberculosis), calcium phosphate, levamisole, isoprinosine, polyanions (e.g., poly A:U), lentinan, pertussis toxin, lipid A, saponins, peptides (e.g., muramyl dipeptide) and rare earth salts (e.g., lanthanum and cerium). The amount of adjuvant depends on the subject and the particular polymer used and can be readily determined by one skilled in the art without undue experimentation. Preferred adjuvants are those that selectively stimulate T cells. It is desirable to avoid adjuvants that might suppress a T cell response.

Other immunomodulators such as cytokines may be delivered in conjunction with the polymers of the invention, and "cocktails" including the polymers and the cytokines are contemplated. The cytokines contemplated are those that will enhance the beneficial effects that result from administering the polymers according to the invention. Cytokines are factors that support the growth and maturation of cells, including lymphocytes. Important to the invention herein is modulating T cell development, as the methods of the invention appear to be T cell-mediated. The cytokines may act directly on T cells or indirectly on T cells through other cells. It is believed that the addition of cytokines will augment cytokine activity stimulated in vivo by carrying out the methods of the invention. The preferred cytokine is interleukin-10, which blocks abscess formation.

Other immunomodulators useful with the polymers of the invention are those that block cytokine activity associated with abscess formation. Certain cytokines such as tumor necrosis factor, interferon and interleukin-2 may participate in abscess formation, since antibodies specific for such substances can help block abscess formation. Such antibodies therefore can be used to supplement the activity of the polymers of the invention. Any immunomodulator which supplements the protective activity of the polymers of the invention can be used.

The precise amounts of the foregoing immunomodulators used in the invention will depend upon a variety of factors, including the polymer selected, the dose and dose-timing



selected, the mode of administration, the nature of any surgery contemplated and the characteristics of the subject. Where local administration is carried out, it will be understood that very small amounts may be required (nanograms and possibly picograms) in the case of cytokines since physiological levels of cytokines are correspondingly low. The precise amounts selected can be determined without undue experimentation, particularly since a threshold amount will be any amount which will favorably enhance the immune response. Thus, it is believed that picogram to milligram amounts are possible, depending upon the mode of delivery, but that nanogram to microgram amounts are likely to be most useful.

The polysaccharides of the present invention have adjuvant properties by themselves. To the extent that the polysaccharides described herein potentiate human immune responses, they can be used as adjuvants in combination with other materials.

Thus, the present invention provides pharmaceutical compositions, for medical use, which comprise polymers of the invention together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients.

The polymers useful in the invention may be delivered separately with another antimicrobial drug or in the form of antimicrobial cocktails. An antimicrobial cocktail is a mixture of any polymer useful with this invention and an antimicrobial drug and/or supplementary potentiating agent. The use of antimicrobial agents in the treatment of bacterial infection is routine. In this embodiment, a common administration vehicle (e.g., tablet, implant, injectable solution, etc.) could contain both the polymer and the antimicrobial drug and/or supplementary potentiating agent. The antimicrobial drug, of course, can be separately dosed.

Antimicrobial drugs are well known and include, but are not limited to, agents from the following classes and agents: sulfonamides, aminoglycosides, quinolones, fluoroquinolones, tetracyclines, penicillins, cephalosporins,  $\beta$ -lactams, macrolides, nitrofurans, metronidazole, clindamycin, lincomycin, vancomycin, rifampin, chloramphenicol, bacitracin, isoniazid, trimethoprim, sulfamethoxazole, polymyxins and spectinomycin. (See Goodman and Gilman's, *Pharmacological Basics of Therapeutics*, 8th Ed., 1993, McGraw Hill Inc.)

The preparations of the invention are administered "in conjunction with" infection, meaning close enough in time with the surgery, trauma or diseases that predispose the host to abscess formation so that a protective effect against abscess formation is obtained. The preparations may be administered long before surgery in the case of elective surgery (i.e.,

weeks or even months) preferably with booster administrations closer in time to (and even after) the surgery. Particularly in emergency situations, the preparations may be administered immediately before (minutes to hours) and/or after the trauma or surgery. It is important only that the preparation be administered close enough in time to the surgery so as to enhance the subject's immune response against bacterial infection/contamination, thereby increasing the chances of a successful host response and reducing the likelihood of abscess formation.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a polymer that will alone, or together with further doses, inhibit or prevent the formation of abscess resulting from infection by a particular bacteria. It is believed that doses ranging from 1 nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, will be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms/kilogram, and most preferably between 1 microgram and 100 micrograms/kilogram. The absolute amount will depend upon a variety of factors (including whether the administration is in conjunction with elective surgery or emergency surgery, concurrent treatment, number of doses and individual patient parameters including age, physical condition, size and weight) and can be determined with routine experimentation. Preferably, a maximum dose is used, that is, the highest safe dose according to sound medical judgment.

Multiple doses of the pharmaceutical compositions of the invention are contemplated. The invention has been shown to be effective with multiple doses administered over a three week period preceding surgery, over a two week period preceding surgery, over a one week period preceding surgery, when the first dose was administered only 24 hours preceding surgery and even when given only after exposure to bacteria. Further doses may be administered post-surgery as well. Any regimen that results in an enhanced immune response to bacterial infection/contamination and subsequent abscess formation may be used, although optimum doses and dosing regimens are those that would not only inhibit the development of abscess formation, but also would result in a complete protection against abscess formation by a particular or a variety of bacterial organisms. Desired time intervals for delivery of multiple doses of a particular polysaccharide can be determined by one of ordinary skill in the art employing no more than routine experimentation.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular polymer selected, the particular condition being

treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are parenteral routes. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intraperitoneal, intrasternal injection or infusion techniques and topical.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the polymer into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the polymer into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. The polymer may be stored lyophilized.

Other delivery systems include timed-release, delayed release and sustained release systems. Such systems can avoid repeated administrations of the polymers of the invention, increasing convenience to the subject and the physician. Such systems are available and known to those of ordinary skill in the art. They include polymer-based systems such as polylactic and polyglycolic acid, polyanhydrides and polycaprolactone; nonpolymer systems that are lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di and triglycerides; hydrogel release systems; silastic systems; peptide based systems; wax coatings, compressed tablets using conventional binders and excipients, partially fused implants and the like. Examples include, but are not limited to: (a) erosional systems in which the polysaccharide is contained in a form within a matrix, found in U.S. Patent Nos. 4,452,775 (Kent); 4,667,014 (Nestor et al.); and 4,748,034 and 5,239,660 (Leonard) and (b) diffusional systems in which an active component permeates at a controlled rate through a polymer, found in U.S. Patent Nos. 3,832,253 (Higuchi et al.) and 3,854,480 (Zaffaroni). In addition, a pump-based hardware delivery system can be used, some of which are adapted for implantation.

### **EXAMPLE 1**

#### **Sources of Bacteria, Isolation and Modification of Polysaccharides, and Preparation of Inoculum**

B. fragilis NCTC 9343 and ATCC 23745, B. distasonis ATCC 8503, and Fusobacterium varium ATCC 8501 were originally obtained from the National Collection of Type Cultures (London, England) or the American Type Culture Collection (Bethesda, MD).

B. thetaiotaomicron 5482 and Enterococcus faecalis 2603 strains were obtained from stock culture collections of the Channing Laboratory, Brigham and Women's Hospital (Boston, MA). Microorganisms were stored at -80°F in peptone-yeast or brain heart infusion broth until used, and grown anaerobically as previously described. Pantosti et al., Infection and Immunity 59:2075-2082 (1991). CPC from B. fragilis NCTC 9343 or ATCC 23745 was isolated by hot phenol/water extraction and subsequent purification of PSA and PSB performed as previously described. Tzianabos et al., Journal of Biological Chemistry 267:18230-18235 (1992).

The S. pneumoniae type 1 capsular polysaccharide (CP) and other pneumococcal polysaccharides were obtained in pure form from the ATCC (MD).

Chemical modifications of polysaccharides to produce molecules with altered charges have been described previously. L. Taylor and H. Conrad, Biochem. 11:1383 (1972) (reduction) and Baumann, A. et al. Biochem. 31:4081 (1992) (N-acetylation and deamination).

Inocula contained a 1:1 mixture of the challenge organism(s) and an adjuvant solution containing sterile rat cecal contents and 10% barium sulfate (w/v) as previously described. Onderdonk, A. et al., Infection and Immunity 13:22-26 (1976). For some experiments, a cecal content inoculum containing feces procured from the ceca of meat-fed rats was used to challenge animals. Onderdonk, A. et al., Infection and Immunity 10:1256-1259 (1974).

The cecal contents inoculum was mixed with barium sulfate (10% final concentration, w/v) and titered in the rat model to yield approximately 50% mortality in a given group of rats with 100% rate of abscess formation in survivors.

## **EXAMPLE 2**

### **Abscess Formation and Immune Response**

#### **Abscess Induction**

The rat model of intra-abdominal sepsis used in this study has been described previously. Onderdonk, A. et al., J. Infect. Diseases 136:82-89 (1977) and Tzianabos, A. et al., Science 262:416-419 (1993). Six days after surgery and challenge, animals were necropsied in a blinded fashion and examined for the formation of one or more intra-abdominal abscesses by an observer blinded to the experimental groups.

The inoculum contained a 1:1 mixture of the test polysaccharide and an adjuvant solution containing sterile rat cecal contents and 10% barium sulfate (W/V) as described in

Tzianabos, A. et al., *Science* 262:416-419 (1993).

A mathematical model was used to compare the biologic activities of modified and unmodified polysaccharides over a range of three doses (200, 20 and 2 µg), and to calculate a dose of each polysaccharide that induced abscesses in 50% of the animals ( $AD_{50}$ ). Reed, L. and H. Muench, *Am. J. Hyg.* 27:493 (1938). Abscesses induced by these polysaccharides were generally uniform in size and those rats that possessed one or more fully formed abscesses were scored as positive. Animals that did not have any fully formed abscesses were scored as negative. Two control groups were included in all experiments: positive controls were challenged with intact *B. fragilis* mixed with adjuvant solution, while negative controls received adjuvant solution alone. In all cases, 100% of the positive control group and none of the negative control group developed abscesses. Data were accumulated from two separate trials.

Different polysaccharides were compared for abscess-forming ability in several experiments. Native PSA was compared with native PSB and the CPC. PSA was an order of magnitude more active ( $AD_{50}=0.67\mu\text{g}$ ) than PSB ( $AD_{50}=25\mu\text{g}$ ) or the CPC ( $AD_{50}=22\mu\text{g}$ ).

The abscess-forming ability of native PSA and PSB were compared with chemically modified versions of PSA and PSB (Table 1).

TABLE 1

Type of Polysaccharide	Fraction of rats with abscess at indicated dose				
	200 µg	20 µg	2 µg	$AD_{50}(\mu\text{g})$	P value*
PSA (native)	16/20	14/20	10/19	1.3	—
PSA (reduced)	5/20	2/19	2/19	>200	<0.0005
PSA (N-acetylated)	7/20	3/19	1/17	>200	<0.0005
PSA (deaminated)	7/20	6/18	3/19	>200	<0.0005
PSB (native)	18/19	13/18	7/19	4	—
PSB (reduced)	17/18	15/20	9/19	3	NS
PSB (N-acetylated)	4/20	5/20	1/20	>200	<0.005
PSB (De-N-acetylated)	9/20	2/20	1/20	>200	<0.005

\*As compared with PSA or PSB (native).

The  $AD_{50}$  of native *B. fragilis* PSA was less than 2 µg. Conversion of the negatively charged carboxyl group associated with the pyruvate substituent PSA to a neutral hydroxymethyl group by carbodiimide reduction (reduced) created a polysaccharide with no

negative charge. This modification of PSA resulted in an increase in  $AD_{50}$  greater than  $200\mu\text{g}$ .

N-acetylation of the free amino group on the trideoxyfucosamine (N-acetylated) of PSA and removal of the same free amino group by nitrous deamination (deaminated) created a polysaccharide with one negative charge and no positive charges per repeat unit. These modifications also significantly reduced the abscess induction by these polymers as compared with that of native PSA so that they displayed an  $AD_{50}$  of greater than  $200\mu\text{g}$ . Modification of the charged groups reduced the biologic potency by at least two orders of magnitude demonstrating that PSA requires both amino (positive) and carboxyl (negative) groups to promote abscess induction in this animal model. Each of the modifications yielded a significant reduction in abscess-inducing ability as compared with that of native PSA ( $P < 0.0005$ ).

Conversion of the negatively charged carboxyl group on the galacturonic acid of the PSB to a hydroxymethyl group via carbodiimide reduction (reduced) did not alter the ability of PSB to induce abscesses as can be evidenced by the  $AD_{50}$  value of  $3\mu\text{g}$ . N-acetylation of the free amino group associated with the 2-aminoethylphosphonate substituent of PSB (N-acetylated) created a polysaccharide with two negatively charged groups (carboxyl and phosphonate) and no positive charges, significantly reducing the abscess induction by this polymer, so that it displayed an  $AD_{50}$  of greater than  $200\mu\text{g}$ .

Replacement of acetyl groups from the three amino sugars present in the repeating unit with three free amino groups created a net positive charge on PSB with a 4:2 ratio of positively to negatively charged groups (de-N-acetylated). The de-N-acetylation of PSB significantly reduced abscess forming potential ( $AD_{50}$  greater than  $200\mu\text{g}$ ). Perhaps the increased number of positively charged amino groups on this polysaccharide prevents critical interactions with cell receptors that initiate the cascade of cellular events resulting in abscess formation.

Some other naturally existing and chemically modified polysaccharides were also assessed for abscess forming ability. The results of these experiments are depicted in Table 2.

TABLE 2

Fraction of rats with abscess at indicated dose					
Type of Polysaccharide	200 µg	20 µg	2 µg	AD <sub>50</sub> (µg)	P value*
C substance	17/18	12/18	6/19	5	—
C substance (N-acetylated)	5/10	1/10	1/10	200	<0.05*
<i>S. pneumoniae</i> Type 1	17/20	7/18	2/18	31	—
<i>S. pneumoniae</i> Type 1 (N-acetylated)	6/19	6/20	3/20	>200	0.018*
<i>S. pneumoniae</i> Type 3	0/14	2/10	1/9	>200	<0.005*
Group B meningococcal	1/10	ND	ND	-	-
Group B streptococcal type Ia	0/10	ND	ND	-	-
Group B streptococcal type III	1/10	ND	ND	-	-
<i>S. pneumoniae</i> type 14	2/10	ND	ND	-	-
Vi antigen	3/20	ND	ND	-	-
Vi antigen (De-N-acetylated)	15/20	9/18	7/20	16	<0.005+

\* compared to native C substance

+ compared to native Vi antigen

The naturally occurring polysaccharides which have oppositely charged groups include, C substance, the group B polysaccharide from *S. pneumoniae*, and the capsular polysaccharide of *S. pneumoniae* strains. C substance has a tetrasaccharide repeating unit with a total of three positive charges (conferred by a phosphatidylcholine substituent and two free amino groups) and two negative charges (conferred by phosphate groups). The capsule of *S. pneumoniae* type 1 has a trisaccharide repeating unit with one positive charge (free amino group) and two negative charges (carboxyl groups). Each was a potent inducer of abscesses, with AD<sub>50</sub> values of 5 and 31 µg respectively. However, when each molecule was N-acetylated to neutralize the free amino group, the result was a marked reduction in abscess-inducing activity. This result was expected for the capsule of *S. pneumoniae* type 1 based on the results obtained in Table 1. However, it was unexpected that C substance would exhibit such a dramatic reduction in activity because it still has a positive charge (conferred by a phosphatidylcholine substituent) and two negative

charges. These results indicate that free amino groups on these polysaccharides are necessary for abscess-inducing activity. A different type of positive charge could not be substituted. No structural requirement exists, however, for a negatively charged group on these polymers.

Naturally occurring bacterial polysaccharides that have repeating unit structures devoid of charged groups such as *S. pneumoniae* type 14 or that have one negatively charged group per repeating unit, such as *S. pneumoniae* type 3, capsular polysaccharides of group B *Neisseria meningitidis* or of types Ia and III of group B streptococci, were tested for ability to form abscesses. The results are shown in Table 2. Each of the above polysaccharides was a poor inducer of abscesses.

Finally, in order to determine whether an inactive polysaccharide which naturally has only a negative charge could be activated, a positive charge was added to the Vi antigen (a homopolymer of galactaminuronic acid from *S. typhi*). Vi antigen, which has an N-acetyl group at the C-2 position of the pyranose ring and a negatively charged carboxyl group at the C-6 position, was modified by alkali treatment to produce a positively charged free amino group and a negatively charged carboxyl group. This chemical modification transformed the antigen into an abscess-inducing polysaccharide.

The data presented in Tables 1 and 2 reveal that abscess induction in the peritoneal cavity of rodents is mediated by oppositely charged groups on bacterial polysaccharides and that a positively charged amino group is required.

### **Immune Response**

Animals were treated with bacterial polysaccharides by subcutaneous injection of 10 $\mu$ g of polysaccharide in 0.1 ml of phosphate buffered saline three times a week for three weeks. Animals received a single treatment on week five and were available for challenge on week six.

The immune response conferring protection against abscess induction was measured after challenge of previously treated animal with homologous and heterologous *B. fragilis* strains as well as other homologous and heterologous bacterial polysaccharides. The results are shown in Table 3. Previous studies have shown that the 9343 CPC and 23745 CPC are distinct polysaccharide complexes. Pantosti, A. et al., *Infect. & Immun.* 59:2075-2082 (1991).

Immunochemical studies have demonstrated that like 9343 CPC, the 23745 CPC consists of at least two distinct polysaccharides possessing positively and negatively charged groups, although the constituent monosaccharides of the CPC found on the two strains are distinct.



Pantosti, A. et al., *Infect. & Immun.* 59:2075-2082 (1991) and Kasper, D. et al., *J. Bacter.* 153:991-997 (1983). Heterologous and homologous *B. fragilis* species were used to challenge rats previously treated with either purified 9343 CPC or 23745 CPC. Treatment with CPC from either source protected rats against abscess formation.

TABLE 3

Polysaccharide Treatment (10 $\mu$ g)	Challenge Polysaccharide (200 $\mu$ g)	Number of rats with abscesses / total	P value
PSA	PSA	3/10	<0.005
PSA	PSB	1/10	<0.005
PSA	<i>S. pneumoniae</i> type 1 PS	3/10	<0.05
PSB	PSA	1/8	<0.005
PSB	PSB	2/10	<0.005
PSB	<i>S. pneumoniae</i> type 1 PS	4/10	<0.05
<i>S. pneumoniae</i> type 1 CP	PSA	2/10	<0.005
<i>S. pneumoniae</i> type 1 CP	PSB	2/10	<0.005
<i>S. pneumoniae</i> type 1 CP	<i>S. pneumoniae</i> type 1 PS	0/10	<0.005

+Each group was compared with animals immunized with unmodified PSA and challenged with this same polysaccharide.

As described above it has been established that particular structural features (free amino and negatively charged groups) on polysaccharides mediate abscess formation. To test whether these polysaccharides confer protection against abscess induction, animals were treated with either PSA or PSB, or the *S. pneumoniae* type 1 CP and challenged with heterologous and homologous polymers. Each polymer protected against abscess formation (Table 3).

In order to determine if the charged groups on these polysaccharides were responsible for mediating protection to abscess formation, chemical modifications of PSA were performed. PSA was modified to neutralize both the positively and negatively charged groups (as described above) and the modified polysaccharide was used to treat animals for protection studies. Animals were treated with N-acetylated PSA to eliminate the positive charges or reduced PSA to eliminate the negative charges and challenged with a native, unmodified PSA. In each case the modified polysaccharides failed to protect animals against polysaccharide-induced abscess

formation demonstrating that the free amino and carboxyl groups in PSA are essential for polysaccharide-mediated protection against abscess formation (Table 4).

TABLE 4

Polysaccharide Treatments (10 $\mu$ g)	Challenge Polysaccharide (200 $\mu$ g)	Number of rats with abscesses/total	P value +
PSA	PSA	1/8	-----
PSA: N-acetylated	PSA	8/10	<0.005
PSA: reduced	PSA	7/10	<0.05
GBS Type 3 CP	PSA	7/9	<0.05
GBS Type 3 CP	PSB	8/9	-----
GBS Type 3 CP	<i>S. pneumoniae</i> Type 1 CP	7/9	
<i>S. pneumoniae</i> Type 3 CP	PSA	6/9	-----
<i>S. pneumoniae</i> Type 14 CP	PSA	6/7	-----

+Each group was compared with animals immunized with unmodified PSA and challenged with this same polysaccharide.

Bacterial polysaccharides that either completely lacked charged groups or have only negatively charged substituents were tested. Animals were treated with the type 3 Group B streptococcal (GBS) CP and challenged with either PSA, PSB, or the *S. pneumoniae* type 1 CP. The type 3 GBS CP, which has one negatively charged group per repeating unit in a terminal sialic acid residue, failed to protect animals against challenge with each of the abscess inducing polymers (Table 4). Animals treated with the type 3 *S. pneumoniae* CP (one negatively charged group per repeating unit) or the type 14 *S. pneumoniae* CP (no charged substituents in its repeating unit) also failed to protect against abscess formation induced by PSA (Table 4).

### EXAMPLE 3

#### Immune Response is T Cell-Dependent

##### **T Cell-Mediated Protection to Polysaccharide-Induced Abscesses**

Animals were treated as described above and were available for adoptive transfer experiments on week 6. Cell transfer experiments were performed as previously described. Shapiro, M. et al., *J. Immunol.* 137:341-346 (1986) and Shapiro, M. et al., *J. Exp. Med.* 154:1188-1197 (1982). Briefly, spleens were removed from treated or naive rats and gently

teased in RPMI medium supplemented with 5% fetal calf serum. Cells were counted using a Coulter FN Counter (Coulter Electronics Inc., Hialeah, FL) and were examined for viability by trypan blue exclusion. The preparation was enriched for T cells by passage over nylon wool columns. Purified T cells were then counted and adjusted to the appropriate cell number ( $1 \times 10^7$ /animal) prior to intra-cardiac transfer to animals (0.2 ml volume). Animals were challenged twenty-four hours later.

It was previously demonstrated that treatment with the purified CPC protects against abscess formation following challenge with viable *B. fragilis* by a T cell-dependent mechanism. Onderdonk, *J. Clin. Investigation* 69:9-16 (1982). In the present experiments, we tested whether protection against polysaccharide-induced abscesses is also T lymphocyte-dependent. Naive rats were administered purified T cells ( $1 \times 10^7$  cells/animal) obtained from animals previously treated with PSA. T cell recipients were then challenged with abscess-inducing PSA, PSB or the type 1 *S. pneumoniae* CP. In each case, T cells from PSA-treated animals protected naive animals against abscesses formed subsequent to homologous and heterologous polysaccharide challenge (Table 5). Rats receiving T cells obtained from animals immunized with type 3 GBS CP (no positively charged group) were not protected against abscesses following challenge with PSA (Table 5).

TABLE 5

Polysaccharide Treatment (10 $\mu$ g)	Challenge Polysaccharide (200 $\mu$ g)	Number of rats with abscesses / total	P value+
saline	PSA	7/8	-----
type 3 GBS	PSA	4/5	-----
PSA	PSA	0/8	<0.05+
PSA	PSB	1/8	<0.05+
PSA	<i>S. pneumoniae</i> type 1 PS	0/7	<0.05+
PSA (N-acetylated)	PSA	7/9	<0.005*
PSA (reduced)	PSA	6/9	<0.05*
Vi	PSA	7/9	-----
Vi (De-N-acetylated)	PSA	2/8	<0.05¶

- + compared with animals given T cells from saline-treated rats and then challenged with PSA
- \* compared with animals given T cells from PSA-treated rats and then challenged with PSA
- ¶ compared with animals given T cells from Vi polysaccharide-treated rats and then challenged with PSA

#### **Chemical Modification to Eliminate Oppositely Charged Groups on Polysaccharides that Induce T Cell-Dependent Protection against Abscess Induction is Eliminated by Chemical Modification of PSA**

To assess the role of the oppositely charged groups on PSA in T cell-mediated protection against abscess induction, animals were treated with either N-acetylated PSA or carbodiimide-reduced PSA and T cells from these animals administered to naive rats. T cells taken from animals treated with the chemically modified versions of PSA failed to confer protection against challenge with unmodified PSA (Table 5).

#### **T Cell-Mediated Protection is induced by a Polysaccharide Manipulated to Contain Oppositely Charged Groups**

Additional adoptive T cell transfer experiments were performed in order to confirm that the presence of both positively and negatively charged groups in polysaccharides are required for protection to polysaccharide-mediated abscess formation. Chemical de-N-acetylation of the Vi capsular polysaccharide of *S. typhi*, a homo-polymer of galactaminuronic acid, was employed to convert this polysaccharide from a polymer that possessed one negatively charged carboxyl group per repeating unit to a saccharide that possessed one positively charged amino group and one negatively charged carboxyl group per repeating unit. In this experiment, T cells were harvested from the spleens of animals treated with the unmodified or de-N-acetylated Vi polysaccharides and transferred to separate groups of naive rats. Each group of rats that received T cells was challenged with *B. fragilis* PSA. Rats receiving T cells from animals treated with the unmodified Vi polysaccharide were not protected against abscess induction by PSA, while animals receiving T cells from rats treated with de-N-acetylated Vi polysaccharide were protected against abscess induction by PSA (Table 5).

#### **EXAMPLE 4**

##### **PSA - mediated Protection against *B. fragilis*-induced Abscesses**

**Effect of regimen.** Several regimens were tested that varied the duration of PSA administration on protection against abscess formation. Animals were administered designated

concentrations of B. fragilis PSA by the subcutaneous route in 0.1 ml of PBS. For some experiments, animals were treated with PSA three times a week for three weeks with an additional dose 24 hours prior to challenge (Regimen 1). To determine the effect of shortening this treatment regimen, further experiments were performed in which Regimen 1 was reduced by one week (Regimen 2) and by two weeks (Regimen 3). A fourth regimen (Regimen 4) included treatment with PSA 24 hours prior to challenge as well as 4 and 24 hours following challenge. With each regimen, groups of animals were given 10 µg/injection of PSA, challenged with B. fragilis and six days later examined for abscess formation. This study shows that PSA protects against B. fragilis infection-mediated abscesses. Irrespective of the length of time animals were treated, fewer animals in each group possessed abscesses when compared to the saline-treated control group ( $P < 0.05$ ). In addition, the protective activity was comparable in each of these groups. For Regimens 1 through 4 the abscess rates were 26%, 18%, 32%, and 12%, respectively, compared with the saline control group, which had an 80% abscess rate.

**Effect of dose.** A dose response experiment was performed using the briefest of the four treatment regimens (Regimen 4). Groups of animals received 10, 1, or 0.1 µg/injection of PSA and were challenged with B. fragilis. In this experiment, significantly fewer animals receiving 10 or 1 µg/injection had abscesses compared with saline-treated control animals (Table 6). However, animals receiving 0.1 µg/injection of PSA were not significantly protected against abscess formation.

**Effect of treatment initiated post-contamination.** To assess the ability of PSA to inhibit abscess induction following intraperitoneal contamination, animals were first challenged with B. fragilis and then treated with varying doses of PSA 1, 24, and 48 hours post-challenge (Regimen 5). Results from this experiment also are shown in Table 6. Treatment of infected animals with 50 µg/injection of PSA yielded a significant level of protection against abscess formation compared with saline-treated controls ( $P = 0.001$ ).

TABLE 6

Treatment Regimen	Dose ( $\mu$ g)	Challenge Inoculum	Abscess Formation (No. rats with abscesses/No. tested)	P value
Saline	-----	<i>B. fragilis</i>	8/10 (80%)	-----
Regimen 4	10	<i>B. fragilis</i>	5/19 (26%)	0.02
Regimen 4	1	<i>B. fragilis</i>	6/19 (25%)	0.02
Regimen 4	0.1	<i>B. fragilis</i>	10/20 (50%)	NS
Regimen 5	50	<i>B. fragilis</i>	3/19 (16%)	0.001
Regimen 5	25	<i>B. fragilis</i>	9/17 (53%)	NS
Regimen 5	10	<i>B. fragilis</i>	6/10 (60%)	NS

**EXAMPLE 5****PSA-mediated Protection against Abscess-inducing Heterologous Bacterial Species**

Following administration of 10  $\mu$ g doses of PSA according to Regimen 1, animals were challenged via the intraperitoneal route with various inocula containing bacteria commonly associated with abscesses found in humans. Groups of animals were challenged with a monomicrobial culture of *B. fragilis*, or mixed inocula consisting of combination of *B. distasonis* and *E. faecalis*, *B. thetaiotaomicron* and *E. faecalis* or *F. varium* and *E. faecalis*. In each case, fewer animals treated with PSA prior to bacterial challenge had abscesses than the saline-treated control group. (Table 7)

TABLE 7

Treatment	Challenge Inoculum	Abscess formation (No. Rats with abscesses/No. Tested)	P value
saline	<i>B. fragilis</i>	15/18 (83%)	-----
saline	<i>B. distasonis</i> + <i>E. faecalis</i>	16/18 (89%)	-----
saline	<i>B. thetaiotaomicron</i> + <i>E. faecalis</i>	15/19 (79%)	-----
saline	<i>F. varium</i> + <i>E. faecalis</i>	13/15 (87%)	----
PSA	<i>B. fragilis</i>	1/19 (5%)	<0.0001

TABLE 7 (cont.)

<u>Treatment</u>	<u>Challenge Inoculum</u>	<u>Abscess formation</u> (No. Rats with abscesses/No. Tested)	<u>P value</u>
PSA	<i>B. thetaiotaomicron</i> + <i>E. faecalis</i>	2/19 (11%)	<0.0001
PSA	<i>F. varium</i> + <i>E. faecalis</i>	5/17 (29%)	<0.002
<i>S. pneumoniae</i> type 3 CP	<i>B. fragilis</i>	9/10 (90%)	NS

**EXAMPLE 6****Protection against Challenge with Cecal Contents**

To simulate fecal contamination of the human peritoneal cavity, an inoculum of cecal contents was surgically implanted into the peritoneal cavity of rats. Although isolated from the ceca of rats, this inoculum contained a microbial flora similar to that found in the human colon. This inocula yielded approximately 50% mortality with all the survivors developing abscesses. Initially, the efficacy of PSA (50 µg/injection according to Regimen 4) against abscess formation by the cecal inoculum was tested. However, only a small reduction in the rate of abscess formation in treated animals was observed. Therefore, another treatment regimen was devised to increase the number of treatments with PSA following implantation of the cecal contents inoculum. In these experiments, animals were treated with 50 µg of PSA 24 hours prior to surgery as well as 4, 24, 48, and 96 hours following surgery. Following this treatment, significantly fewer animals developed abscesses compared with animals treated with saline (12/25 versus 18/18, 48% versus 100%,  $P < 0.0001$ ). This establishes that PSA shows broad protection against multiple organisms found in cecal contents.

**EXAMPLE 7****T cell-mediated Protection against Abscess Formation.**

It was investigated whether cell-mediated immune mechanisms controlled the broadly protective activity exhibited by PSA in abrogating abscess formation by heterologous organisms commonly associated with intra-abdominal sepsis. Animals were treated with PSA (10 µg/injection) according to Regimen 3 and T cells isolated 24 hours after last treatment. T cells ( $1 \times 10^7$ ) from PSA-treated or saline-treated animals were administered to naive recipients 24

( $1 \times 10^7$ ) from PSA-treated or saline-treated animals were administered to naive recipients 24 hours prior to challenge with *B. fragilis* or with a mixed inoculum of *F. varium* and *E. faecalis*. Results are shown in Table 8. Animals receiving T cells from saline-treated rats and challenged with *B. fragilis* or the *F. varium* and *E. faecalis* combination developed abscesses (85% and 87%, respectively). However, transfer of T cells from PSA-treated animals to naive recipients yielded significant protection against abscess formation following challenge with *B. fragilis* (13% abscess rate) or *F. varium* and *E. faecalis* (21% abscess rate).

TABLE 8

Treatment (10 $\mu$ g)	Challenge Inoculum	Abscess-Formation (No. rats with abscesses/No. tested)	P value
saline	<i>B. fragilis</i>	11/13 (85%)	-----
saline	<i>F. varium</i> and <i>E. faecalis</i>	13/15 (87%)	-----
PSA	<i>B. fragilis</i>	1/8 (13%)	< 0.005
PSA	<i>F. varium</i> and <i>E. faecalis</i>	3/14 (21%)	< 0.005

Those skilled in the art will be able to recognize or ascertain with no more than routine experimentation numerous equivalents to the specific products and processes described above. Such equivalents are considered to be within the scope of the invention and are intended to be covered by the following claims in which we claim:



**CLAIMS**

1. A method for inducing protection against abscess formation associated with infection comprising: administering to a subject in need of such protection a pharmaceutical preparation containing an effective amount for inducing protection against abscess formation of a polymer of repeating units of a charge motif characteristic of polysaccharide A, the motif being a positively charged free amino moiety and a negatively charged moiety selected from the group consisting of carboxyl, phosphate, phosphonate, sulfate and sulfonate, and wherein the polymer is free from complexation as part of a B. fragilis capsular polysaccharide complex.
2. The method of claim 1 wherein the pharmaceutical preparation induces protection against abscess formation by at least two microorganisms selected from the group consisting of Bacteroides, Clostridia, Fusobacteria, Enterococcus, Prevotella, Porphyromonas, Staphylococcus, Propionobacteria, Peptostreptococcus, Streptococcus, Veillonella, Actinomycetes, and E. coli.
3. The method of claims 1 or 2 wherein the pharmaceutical preparation is administered to a subject who is in need of surgery.
4. The method of claims 1-3 wherein the polymer is a polysaccharide formed by the process of de-N-acetylation of a precursor polysaccharide.
5. The method of claims 1-3 wherein the polymer is a polysaccharide formed by the process of reducing an imine group of a precursor polysaccharide.
6. The method of claims 1-5 further comprising co-administering to the subject an amount of an antimicrobial drug or an immunomodulator effective for enhancing the induction of protection against abscess formation.
7. The method of claims 1-6 wherein each repeating unit is a polysaccharide of a maximum of ten saccharides.

8. The method of claims 1-6 wherein each repeating unit is a polysaccharide of a maximum of five saccharides.
9. The method of claims 1-8 wherein the polymer is a bacterial capsular polysaccharide.
10. The method of claims 1-6 wherein the polymer is polysaccharide A of B. fragilis.
11. A method for preparing a pharmaceutical comprising:
  - selecting a polymer having repeating units each with a maximum of ten units, each unit having at least one negatively charged moiety selected from the group consisting of carboxyl, phosphate, phosphonate, sulfonate and sulfate, and having at least one moiety capable of being modified to produce a positively charged amino moiety;
  - modifying the polymer to produce at least one free positively charged amino moiety on each repeating unit, and
  - combining the modified polymer with a pharmaceutically acceptable carrier to form a pharmaceutical composition for protecting a subject against abscess formation associated with infection.
12. The method of claim 11 wherein the polymer is a polysaccharide and the at least one moiety is an N-acetyl moiety, and wherein the polysaccharide is modified by de-N-acetylation to convert the at least one N-acetyl moiety of each repeating unit to a free amino moiety.
13. The method of claim 11 wherein the polymer is a polysaccharide and the at least one moiety is an imine group, and wherein the polysaccharide is modified by reduction to convert the at least one imine group of each repeating unit to a free amino moiety.
14. The method of claims 11-13 wherein the polysaccharide is a bacterial capsular polysaccharide.

15. The method of claims 11-14 further comprising combining the modified polysaccharide with an immunomodulator for enhancing the induction of protection against abscess formation or with an antimicrobial drug.
16. A pharmaceutical preparation prepared according to the method of claims 10-14.
17. A pharmaceutical preparation for protecting a subject against abscess formation associated with infection comprising:
- a polymer formed of repeating units each with a maximum of ten units, each repeating unit including a free amino moiety and a negatively charged moiety, selected from the group consisting of carboxyl, phosphate, phosphonate, sulfate and sulfonate; and
  - a pharmaceutically acceptable carrier, wherein the polymer is free from complexation as part of a B. fragilis polysaccharide complex, except that the polymer is not selected from the group consisting of S. pneumoniae polysaccharide, T. cruzi lipopeptidophosphoglycan and P. aeruginosa Fisher immunotype 7 O-antigen.
18. The pharmaceutical preparation of claim 17 wherein the negatively charged moiety is selected from the group consisting of carboxyl, phosphate and phosphonate and wherein the polymer is a polysaccharide.
19. The pharmaceutical preparation of claim 17 wherein the polymer is B. fragilis capsular polysaccharide A free of complexation with B. fragilis capsular polysaccharide B or modified B. fragilis capsular polysaccharide A, modified to contain a hydroxymethyl group.
20. The pharmaceutical preparation of claim 17 wherein the polymer is B. fragilis capsular polysaccharide B, free of complexation with B. fragilis capsular polysaccharide A.
21. The pharmaceutical preparation of claims 17 or 18 wherein the repeating unit is selected from the group consisting of:
- (1) a monomer carrying both the free amino group and the negatively charged group;
  - (2) a complex of a first and a second covalently linked monosaccharide in a 1-4

linkage and wherein the negatively charged group is on the first saccharide;

(3) a trimer of a first, second and third saccharide and wherein the negatively charged moiety is on the first monosaccharide;

(4) a trimer of a first, second and third saccharide and wherein the third saccharide is free of any amino or negatively charged moiety;

(5) a pentamer with a trimeric backbone, characteristic of B. fragilis capsular polysaccharide A; and

(6) a hexamer with a trimeric backbone, characteristic of B. fragilis capsular polysaccharide B.

22. The pharmaceutical preparation of claims 18, 19, and 21 wherein the polymer is a bacterial polysaccharide.

23. The pharmaceutical preparation of claim 17, wherein the pharmaceutically acceptable carrier is constructed and arranged as a sustained release delivery system.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/11160

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROCEEDINGS OF THE 1994 MEETING OF THE ANAEROBE SOCIETY OF THE AMERICAS, MARINA DEL REY, CALIFORNIA, USA, JULY 29-31, 1994. CLINICAL INFECTIOUS DISEASES 20 (SUPPL. 2). 1995. S132-S140., TZIANABOS A O ET AL 'Structure and function of Bacteroides fragilis capsular polysaccharides: Relationship to induction and prevention of abscesses.' see the whole document</p> <p style="text-align: center;">--- -/--</p>	1,2, 11-23

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

12 December 1995

Date of mailing of the international search report

08.01.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+ 31-70) 340-3016

Authorized officer

Moreau, J

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/11160

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 343438, TZIANABOS A ET AL 'Structural basis for polysaccharide -mediated protection against intraabdominal abscess formation.' see abstract &amp; 94TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, LAS VEGAS, NEVADA, USA, MAY 23-27, 1994. ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY 94 (0). 1994. 65. ISSN: 1060-2011, ---</p>	1,2, 11-23
X	<p>INFECT. IMMUN. (1994), 62(8), 3590-3 , 1994 TZIANABOS, ARTHUR O. ET AL 'Structure-function relationships for polysaccharide -induced intra-abdominal abscesses' see the whole document ---</p>	1,2, 11-23
A	<p>SCIENCE (WASHINGTON, D. C., 1883-) (1993), 262(5132), 416-19 , 1993 TZIANABOS, ARTHUR O. ET AL 'Structural features of polysaccharides that induce intra-abdominal abscesses' see the whole document ---</p>	1-23
A	<p>INFECT. IMMUN. (1991), 59(6), 2075-82 , 1991 PANTOSTI, ANNALISA ET AL 'Immunochemical characterization of two surface polysaccharides of Bacteroides fragilis' see the whole document ---</p>	1-23
A	<p>BIOCHEMISTRY (1992), 31(16), 4081-9 , 1992 BAUMANN, H. ET AL 'Structural elucidation of two capsular polysaccharides from one strain of Bacteroides fragilis using high-resolution NMR spectroscopy' see the whole document ---</p>	1-23
A	<p>J. BIOL. CHEM. (1992), 267(25), 18230-5 , 1992 TZIANABOS, ARTHUR O. ET AL 'The capsular polysaccharide of Bacteroides fragilis comprises two ionically linked polysaccharides' see the whole document ---</p>	1-23
	-/--	

1

# INTERNATIONAL SEARCH REPORT

I. International Application No  
PCT/US 95/11160

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>INFECT. IMMUN. (1994), 62(11), 4881-6, November 1994 TZIANABOS, ARTHUR O. ET AL 'Structural characteristics of polysaccharides that induce protection against intra-abdominal abscess formation' see the whole document -----</p>	1-23

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/11160

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-10  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.